ISOLATION AND IDENTIFICATION OF ACETIC ACID PRODUCER FROM SOIL USING BANANA STEM WASTE AS A SUBSTRATE

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ABSTRACT

This research dealt with the identification of acetic acid producer (AAP) from soil mixed culture (SMC), production of acetic acid from banana stem waste (BSW) and kinetic study of the acetic acid production. 57 bacterial strains were isolated from SMC. From the isolation, 15 strains produced acetid acid. Later, only five most efficient bacterial strains were identified using biochemical test and biomolecular test. Those five strains were labeled as A1, A2, A6, A7, and A8. All isolated strains were in rod shape and the colonies size ranged was from 1.0mm to 3.0mm. A1 was identified as *Bacillus cereus PG06* with 92% match, A2 as *Klebsiella pneumonia ST258* with 92% match, A6 as *Bacillus thuringiensis BMB171* 90% match, A7 as *Bacillus sp. SJ1* with 77% match and A8 as *Enterobacter cloacae Y219* with 89% match. *Bacillus thuringiensis BMB171* (A6) is the most productive bacteria that produced resulted in highest yield of acetic acid (up to 4.4 g/l) compared to other identified strains. Later, kinetic study was conducted using this strain. The experiment was conducted using BSW and glucose medium (as control substrate) in batch fermentation for 72 hours. The proposed kinetic model have 7 kinetic constants (k_1-k_7) which determined based on the sum squared error between experimental data and theoretical data by using Microsoft Excel. The initial concentrations of biomass (S_{VSS}), glucose (S_{VDS}), and acetic acid (S_{TAA}) in the calculation were: $(S_{VSS})_0$ = 0.0002 g/l; $(S_{VDS})_0$ = 2.46 g/l; $(S_{TAA})_0$ = 0 g/l. The k_1 is the kinetic constant of the reaction, k_2 is the maximum rate of glucose uptake for growth, k_4 is the maximum rate of glucose uptake for acetic acid production, k_6 maximum rate of acetic acid consumption, and k_3, k_5, k_7 is the saturation constant. The values of calculated kinetic constants were as followed: k_1 = 0.5004 h^{-1}; k_2 = 0.0576g /l h; k_3 = 0.6197g /l; k_4= 0.1294g /l h; k_5= 0.9289g /l; k_6= 0.0545g /l h; k_7= 0.0001g /l. The kinetic constant of substrate utilization and production for BSW was lower than using glucose as medium. This is due to secondary metabolism. In comparison of kinetic study from other researchers show that, the k_1 rate were higher than other research however other kinetic constant were in acceptable range. This is due to the nutrient supplied promoted the bacteria growth. The R^2 values obtained from kinetic study were in acceptable range (above 0.8). Overall, the *Bacillus thuringiensis BMB171* (A6) was the most productive acetic acid producer from soil and, the proposed kinetic model could be used to describe the process.

ABSTRAK

Penyelidikan ini berkaitan dengan pengenalpastian penghasil asetik asid (AAP) dari kultur campuran tanah (SMC), penghasilan asetik asid dari sisa batang pisang (BSW) dan kajian kinetik penghasilan asetik asid. Dari proses pemencilan, 15 strain berjaya menghasilkan asetik asid. Hanya lima strain yang terbaik dikenalpasti menggunakan ujian biokimia and biomolekular. Lima strain tersebut dilabel sebagai A1, A2, A6, A7 and A8. Kesemua strain diasingkan berbentuk rod dan koloni saiz sebesar 1.0mm hingga 3.0mm. A1 dikenalpasti sebagai *Bacillus cereus PG06* dengan 92%, A2 sebagai *Klebsiella pneumonia ST258* dengan 92%, A6 sebagai *Bacillus thuringiensis BMB171* dengan 90%, A7 sebagai *Bacillus sp. SJ1* dengan 77%, dan A8 sebagai *Enterobacter cloacae Y219* dengan 89%. *Bacillus thuringiensis BMB171* (A6) merupakan strain terbaik yang berjaya menghasilkan paling banyak asetik asid (4.4 g/l) apabila dibandingkan dengan strain yang lain. Bakteria ini dipilih untuk kajian kinetik. Uji kaji ini dijalankan dengan menggunakan BSW dan glukosa (substrat kawalan) dalam fermentasi berkelompok selama 72 jam. Model kinetik yang dipilih mempunyai 7 pemalar kinetik (k_1-k_7) yang diperolehi menggunakan perbezaan jumlah ralat kuasa dua antara data eksperimen dan data teori dengan menggunakan Microsoft Excel. Nilai kepekatan awal biomas (S_{VSS}), glukosa (S_{VSS}), dan asetik asid (S_{TAA}) yang digunakan diperolehi dari media BSW adalah seperti berikut: $(S_{VSS})_0$ = 0.0002 g/l; $(S_{VDS})_0$ = 2.46 g/l; $(S_{TAA})_0$ = 0 g/l. k_1 merupakan pemalar kinetik bagi tindakblas, k_2 merupakan pemalar bagi kadar maksima pengambilan glukosa ketika pertumbuhan, k_4 pemalar bagi kadar maksima pengambilan glukosa ketika penghasilan asetik asid, k_6 merupakan pemalar bagi kadar maksima pengunaan asetik asid, dan k_3,k_5,k_7 merupakan pemalar ketepuan. Nilai pemalar yang diperolehi adalah seperti berikut: k_1 = 0.5004 h^{-1}; k_2 = 0.0576g /l h; k_3 = 0.6197g /l; k_4= 0.1294g /l h; k_5= 0.9289g /l; k_6= 0.0545g /l h; k_7= 0.0001g /l. Pemalar kinetik penggunaan substrat dan penghasilan untuk BSW lebih rendah dari glukosa. Perbezaan ini disebabkan oleh metabolisma kedua. Hasil dari perbandingan antara kajian menunjukkan bahawa pemalar k_1 lebih tinggi dari kajian lain, manakala pemalar kinetik lain berada dalam kadar bersesuaian. Ini disebabkan oleh nutrisi yang dibekalkan menggalakkan pertumbuhan bakteria. Nilai R^2 yang diperolehi turut berada dalam kadar bersesuaian (melebihi 0.8). Kesimpulanya, *Bacillus thuringiensis BMB171* (A6) merupakan penghasil asetik asid dari tanah yang paling produktif dan model kinetik yang digunakan sesuai untuk menjelaskan proses yang terlibat.

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h	Hour
min	Minutes
L	Liter
ml	Milliliters
M	Mole
℃	Degree Celsius
pH	Potential Hydrogen
µl	Microliter
%	Percentage
g	Gram
ml	Mililiter

LIST OF ABBREVIATIONS

AAP	Acetic Acid Producer
BSW	Banana Stem Waste
SMC	Soil Mixed Culture
NA	Nutrient agar
NB	Nutrient Broth
PCR	Polymerase Chain Reaction
OD	Optical Density
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomes Ribonucleic Acid
rpm	Revolutions per minute
HPLC	High Performance Liquid Chromatography

CHAPTER 1

INTRODUCTION

This chapter gives the fundamental information of the research. It is divided into the subtopics of background of study, problem statement, research objectives, scope of study, and thesis organization.

1.1 BACKGROUND OF STUDY

The agriculture activity is becoming one of the most important industries in Malaysia. The expansion of agricultural activity in Malaysia gives a lot of advantages by increasing the economic and development in rural area. But, there are also disadvantages where too many agricultural wastes were produced. Malaysia peninsular only, produced up to 4.2 million tons of crop residue (USM RCE, 2006). Usually, the wastes are not treated and left on the fields, discarded into the rivers or treated by open burning by the settlers and farmers. So, it is becoming increasingly difficult to ignore that the agricultural wastes are one of major organic pollutants that polluting Malaysia's rivers and air (USM RCE, 2006).

The agricultural waste or crops can be considered as renewable alternative feedstock than fossil fuel (petroleum). One of the largest agricultural wastes in Malaysia is banana stem waste.

Banana remains as the second most important fruit crop after durian in Malaysia. Banana stem waste, abundantly available in banana production fields and market, appears to be a favorable substrate for acetic acid production as it cheaply and easily to get in the tropical and subtropical countries. According to Baharudin (2009), in every 60 kg of banana grown, almost 200 kg of waste stem are thrown away. Banana plant is in Musacae family, and it is a monocotyledon. Banana stem waste is lignocelluloses, which consist of lignin, cellulose and hemicellulose. It consists of 15.42% lignin, 53.45% cellulose and 28.56% hemicellulose (Silveira et al., 2007). This data shows that high cellulose content can be recovered from banana stem. Thus, it is very suitable to be feedstock for biomass energy and other products such as for acetic acid production.

Acetic acid is one of the organic acids. This high demand material is widely used in industrial application. It is applied in production of plastic monomer, in food industry as vinegar or food additives and few in pharmaceutical industry. There are two fermentation processes for production of acetic acid which are aerobic or anaerobic process. Acetic acid is carbon sources for acetoclastic methanogen, which convert acetate from acetic acid to produce methane (Naidoo et al., 2002). Zainol and Rahman (2009) found that the reaction to produce methane occurred between mixed cultured from a soil sample with banana stem waste in anaerobic condition.

There is also a need to find a potential strain of bacteria that can directly convert the waste to acetic acid from soil. It was cost effective and highly feasible. Specific types of bacteria only involved in specific process. It is due to the product from the process may become toxic to the bacteria. In several cases, some species may involve in several processes such as *Enterobacter sp.* Common isolation method by using selective agar is not viable for this strain. Isolation of acetic acid producer (AAP) from soil and kinetic study of the most efficient AAP were conducted in this study.

1.2 PROBLEM STATEMENT

Some of the criteria to select raw material for production of acetic acid via fermentation process are the material must contained high amount cellulose, abundantly, and not expensive (Busche, 1991; Ebner et al., 1996; Ravinder et al., 2001; Veny and Hasan, 2003). However, there has been little discussion about banana stem waste (BSW) as raw material for production of acetic acid.

Bacteria play an important role in the fermentation process. Besides, the research to date has focused more on aerobic fermentation rather than anaerobic fermentation. Aerobic fermentation of glucose to acetic acid involved two stages process, which are conversion of substrate into ethanol followed by ethanol oxidation into acetic acid. Therefore, this process needs two reactors which increased the cost (Veny et al., 2003; Veny and Hasan, 2005). Furthermore, researches nowadays are dealing with commercial bacteria. Those bacteria are expensive and hard to manage (Song et al., 2010; Veny et al., 2003).

Hence, there is a need to find anaerobic AAP that can produce higher yield, convert complex substrate directly to acetic acid, easy to obtain, easy to handle and cheap. Besides, Zainol and Rahman (2008) found that mixed culture from soil have the ability to produce acetic acid. Hence, soil is a good source to find the pure strains that can replace the aerobic AAP. The potential bacteria such as *Clostridium sp.*, *Aeromonas sp.*, *Syntrophomonas sp.*, and *Enterobacter sp.* can be obtained from soil (Hatamoto *et al.*, 2007; Manter et al., 2010; Lee et al., 2009; Song et al., 2010; Sousa *et al.*, 2007; Zhang *et al.*, 2004).

Kinetic study is needed in order to understand the performance of the system. It is necessary for balanced and rational design of a biological waste-treatment system to forecast the system stability, quality of effluent (Garcia-Ochoa et al., 1999). In some process different type of bacteria will affect the kinetic model used due to some bacteria can secrete several types of enzymes. This may affect the kinetic constant and R^2 value. Therefore, it is important to find the suitable model for specific bacteria. The most common model is Monod model, due to the flexibility of the model. The model implements the effect of a growth limiting nutrient on the specific growth rate. The relation between growth rate, substrate concentration and concentration of product is used in order to understand the process.

1.3 RESEARH OBJECTIVE

There are two main objectives in this research,:

1. To isolate and identify bacteria from soil involved in banana stem waste (BSW) fermentation
2. To determine the kinetic parameter for acetic acid production via BSW fermentation.

1.4 SCOPES OF THE STUDY

The scope of the study is based on the research objective above:

1. To isolate and identify bacteria from soil involved in banana stem waste (BSW) fermentation
    a. To isolate anaerobic bacteria from soil containing banana stem waste as substrate.
    b. To identify anaerobic bacteria that produces acetic acid using banana stem waste via fermentation.
    c. To identify the most efficient anaerobic bacteria that produces acetic acid.

2. To determine the kinetic constant for acetic acid production using BSW via fermentation.
    a. To apply the available kinetic model for acetic acid production using BSW via fermentation.
    b. To use the highest acetic acid producer in the kinetic study.

1.5 THESIS ORGANIZATIONS

This thesis consists of five main chapters. Chapter 1 is outline of the study on isolation of AAP from soil mixed culture and kinetic study in general. For chapter 2, will be describing the literature reviews of every scope covered in this study. Then, the methodology, apparatus and equipment works are discussed in Chapter 3. In addition, the experimental results are discussed in Chapter 4. Finally, chapter 5 concludes the study and consists of suggestion for future research. This thesis is completed with references and appendices.

CHAPTER 2

LITERATURE REVIEW

2.1 ACETIC ACID

Acetic acid, also known as ethanoic acid (CH_3COOH) is one of many types of organic acids. It is the main component in vinegar and has a unique sour taste and strong smell. Industrially, acetic acid is widely used in a production of plastic monomer and reagents for pharmaceutical industries (Hosea et al., 2005).

2.1.1 Acetic acid in Food Industries

Acetic acid in food industries is commonly known as vinegar. It is commonly produced by the fermentation process and regularly used for pickling processes, vinaigrettes, salad dressings, and component in sauces such as mustard, ketchup and mayonnaise (Das and Sarin, 1938). The vinegar is typically produced from raw food such as fruit, vegetables and even fishes. Local people in Malaysia usually produce vinegar using rice, coconut water and nypa water (nira Nipah). In a western country, the vinegar is produced from wine, cider, balsamic, raisin, cane and other fruit juices (Hill et al., 2005).

2.1.2 Acetic acid in Pharmaceutical Industries

Acetic acid can also be used for medical purposes. Prophet Muhammad which lived about 1,400 years ago stated that the best condiment is vinegar (Sahih Muslim, a). He also stated that vinegar is a comfort for man (Sahih Muslim, b), which can be said that, vinegar is good for health. Avicenna who wrote ‘The Canon of Medicine', and Ibn Qayyim Al-Jawziyya claimed that vinegar is a powerful clotting agent. It helps in healing skin burns, gastric inflammations, heartburns and also prevents the effects of toxic medications or poisonous mushrooms. Besides that, the effect of using vinegar is it relieves headaches caused by heat, and prevents tumour from occurring.

Furthermore, it can significantly lower values for serum total cholesterol and triacylglycerol, which will reduce the risk of fatal ischemic heart disease (Fushimi et al, 2006; Johnston et al, 2006). Vinegar is also used to promote lysis of red blood cells in order to do manual white blood cell counts. Moreover, it is applied for cervical cancer screening tool and antimicrobial uses due to its acidity (Szarewski, 2007; Medina et al., 2007).

2.1.3 Acetic acid in other application

Instead of vinegar, acetic acid also can be found in the form of glacial acetic acid. Glacial acetic acid is 99% pure acid which is dangerously corrosive. It is an important reagent used to produce plastic drinking bottles, cellulose acetate for photographic film and polyvinyl acetate for wood glue (Cedar Petrochemicals, 2011). Other than that, acetic acid also acts as a solvent for various applications. Acetophenone is one of the solvent produce from acetic acid and used for the making of alcohol-soluble resins via dry distillation process with calcium salts, acetic acid and other acids. Then, the resins are applied in a whole range of perfumes.

2.1.4 Acetic acid market demand

Acetic acid is a global product. China, United States of America, the rest of Asia and Western Europe are largest acetic acid consumer. The annual worldwide demand of acetic acid is around 6.5 million tonnes of which, approximately 1.5 million tonnes are produced by a fermentation process (Starcontrol, 2011). According to Malveda and Funada (2010), from the entire global acetic acid capacity (virgin acid), 65% is in the Asia where China is the major capacity, 19% in the United States of America and the rest is from other regions. These two regions make up to 84% of whole world capacity. Moreover, China is the largest consumer with 30% of total demand. The rest of Asia accounted for 27% of global demand including Malaysia, with 1 to 3% acetic acid consumption in the world, which is nearly 0.12 million tons acetic acid per annum, followed by 20% in the United States of America and 14% in Western Europe. These regions totalled over 91% of worldwide acetic acid consumption (Malveda and Funada, 2010).

In addition, acetic acid is an important feedstock for production of plastic monomer such as vinyl acetate monomer (VAM) and terephtalic acid (TPA) which holds about 80% of world consumption. In Malaysia, 92,000 tonnes acetic acid per year is needed as raw material to produce TPA, which is locally produced at Kerteh, Terengganu and generates almost 535,000 tonnes acetic acid per annum. Most of the production of acetic acid in Malaysia is exported to China. Although China is the main acetic acid consumer and also the world major plastic producer, they could not afford to fulfil their own demand due to the limited resources in China, hence the need to import them from other countries. Malaysia is known as one of the top supplier acetic acids for China’s market, which contributes 10 - 20% total import of acetic acid in China (IHS chemicals, 2012).

The acetic acid market will continue to grow due to the expansions of VAM and TPA quantity in the Asia region, especially in China and Middle East (Goliath, 2006). Due to high demand but low production or supply around the world, the price for acetic acid is really expensive which cost almost RM 3500.00 per tonnes (Lee and Shen, 2011). Because of the total global acetic acid demand that is massively increasing per year, an alternative method to produce acetic acid need to be introduced. The usage of non-renewable source can be substitute with renewable source because non-renewable source will be depleted. Biological process was introduced as an alternative method that can utilize the renewable source in acetic acid production.

2.2 RAW MATERIALS (LIGNOCELLULOSE MATERIALS)

Raw material is the unprocessed items that are broken down, undergo process or combine with some materials to create a finished product (Roland et al., 2011). In a biological process such as production of acetic acid, the raw materials used are usually natural resources or biomaterials. Few examples of biomaterials are waste from banana stem, corn plant, yam plant and renewable raw materials such as households waste, municipal waste, paper mills and many others. This diversity of biomass can be used to convert cellulose, and then into acetic acid (Okonko et al., 2009). The recycling industry has long started in producing raw materials out of other products.

2.2.1 Banana Stem Waste (BSW)

Banana is a herbaceous perennial of the genus Musa (family Musaceae) that grows in high humidity weather like tropical and subtropical climates. Malaysian tropical rain forest is well known as the centre of origin for this genus. Banana is a main crop of many regions generating a huge pile of waste after harvesting (Roux et al., 2008). Most of the fruits were consumed by local people and about 10% are exported to Singapore, Brunei, Hong Kong and Middle East (Pawiro, 2008). The banana waste, being abundantly available, easy to get and awfully inexpensive, appears to be a favourable substrate for cellulose production.

This agro waste is used as the substrate to produce cellulose in a single step fermentation process. The stem being the most valuable part in a banana tree is mainly composed of 53.45% cellulose, 28.56% hemicellulose and 15.42% lignin that come together as lignocelluloses (Silveira et al., 2008; Elanthikkal et al., 2010). Lignocelluloses are a complex material of rigid cellulose fibers surrounded in a cross-linked medium of lignin and hemicellulose. This shows that the cellulose content that to be recovered from banana stem is high.

2.2.2 Corn Stalk Waste

Dracaena fragrans also known as Corn is a flowering plant. It is a type of food for the livings. Under the family name of *Asparagace*, it is widely grown as office plants and houseplants. Native to western Africa, *Agave* is also grown as ornamentals in tropical climates. These plants were popular because of only minimal maintenance were needed. According to Indexmundi (2012), Malaysia produced up to 100,000 MTA in 2011 and its growth rate was increased up to 5% from the previous year.

According to Chahal et al., (1990), stalks remaining after it was harvested contain 43% polysaccharides consisting mainly of hemicellulose, 29% cellulose, 7% lignin, 5% ash, and 16% others. Cellulose is the main component in plant cell walls. It gives plants structure and provides them with strength and stability. It is also the basis of grasses, woods, and plants stalks, and accounts for 44% of all plants' biomass. Corn stalks are one of them.

In order to obtain acetic acid, the cellulose needs to break down using hydrolysis process. The hydrolysis process breaks the cellulose molecules into its basic sugar form such as glucose and other pentose sugar. This sugar is utilized by microbes, which then would produce acetic acid. The acetic acid will be produced along with the other sub-products such as sulfuric acid that will reduce the acetic acid quality (Guo et al., 2011).

2.2.3 Yam Waste

Yam plant is a monocot colic root with a twining and tuberous vine. Most of the Yam species came from the same genus *Dioscorea*, where 600 wild species that grow well in damp woodlands and thickets, but not all them contain Diosgenin (Coursey, 1967;

Davidson 1999; Kiple et al., 2000). Wild yam usually has a perennial vine with pale brown, knotty, woody cylindrical rootstocks, or tubers. The roots are dry, narrow, and crooked. Its stem can grow to a length of over 30 feet. This differs with sweet potato where sweet potato is a dicot plant and has much complex structure, and also, the Yam root has unique taste (Dhir et al., 2009; Schultheis and Wilson, 1993). The wild yam plant has clusters of tiny, greenish-white and greenish-yellow flowers. The heart-shaped leaves are extended, wide and long-stemmed. The upper surface of the leaves is smooth while the underside is furry (Asiedu et al., 1997). The dietary fiber, non-cellulose polysaccharides, cellulose and lignin content in yam reported to be 13.2%, 9.7%, 2.9% and 0.1%, respectively (Akingbala, 1995; Adamson, 1985). It is reported that waste from yam peels and leaves contained high amount of cellulose which is 40.7% and 13.7% hemicelluloses (Akinfemi et al., 2009).

2.2.4 Cassava Waste

Cassava (*Manihot esculenta Cranz*) or tapioca is one of the important sources of food in tropical countries such as Asia, Africa and South America. It is considered to be originated from South America. It produces tubers and made up from two parts, which are aboveground and underground. The above ground part can be as high as 2-4 m with a trunk and branches on it. The underground part consists of two types of roots which one is responsible for the plant nutrition, while others with axial outlook surrounding the trunk. These parts are called tubers and are the parts that commonly used for food. Each plant may have 5-20 tubers, which may reach a length of 20-80 cm and 5-10 cm diameter (Pandey et al., 2000).

According to Howeler (2002), Malaysia produces 430,000 tonnes from 41,000 hectare around Malaysia. Almost 1.16 tonnes peels, 42 tonnes cassava bagasse from 300 tonnes cassava root used were produced. These wastes are usually left as it is in the environment without any treatment (Pandey et al., 2000). The cassava peels and bagasse contained of 33.2% cellulose and 21% hemicelluloses (Akinfemi et al., 2009).